FLUORESCENCE STUDY OF THE BINDING DYNAMICS OF SACCHARIDES TO LYSOZYMES

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1. Introduction

The complete structure determination of hen egg-white lysozyme and of some lysozyme-saccharide complexes have provided a basis for a better understanding of the mechanism of action of this enzyme in molecular detail [1]. Though a large number of studies have recently been dealing with this problem [2], most of them are concerned with equilibrium properties of the enzyme whereas information on its dynamic behaviour is still limited. Chipman and Schimmel [3] were the first to apply the relaxation technique [4] for the investigation of the dynamics of binding of *N*-acetyl-D-glucosamine (GlcNAc) oligomers to lysozyme. They followed the proton transfer reaction resulting from the binding process, using a pH indicator and concluded that the binding of $(GlcNAc)_2$ or $(GlcNAc)_3$ to lysozyme is a simple bimolecular process. Holler, Rupley and Hess [5] examined the same system over a wider concentration range and obtained evidence for a more complex binding mechanism involving two steps: a fast step of association between saccharide and lysozyme followed by a slow isomerization of the enzyme-saccharide complex.

In this report, we describe results of an investigation of the dynamics of binding to lysozyme of the chitin oligosaccharides $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_5$ using the temperature-jump method. The binding reactions were followed by observing the changes in the fluorescence of the enzyme-saccharide system. A marked advantage of this approach is that the fluorescence changes can be correlated with changes in some specific regions of the active site of the enzyme, in particular of Trp-108 [6]. The experimental data are interpreted in terms of a binding mechanism involving several steps. Identical kinetic and equilibrium parameters were found for all three oligosaccharides investigated. In addition, evidence is presented for the involvement of Trp-108 and Glu-35 in a saccharide induced isomerization of lysozyme.

2. Materials and methods

Hen egg-white lysozyme, twice recrystallized, salt free (LYS F 9BD) from Worthington was used throughout this study. Iodine oxidized lysozyme in which Trp-108 is modified by oxidation of its indole ring into oxindole was prepared according to Hartdegen and Rupley [7] and lyophilized. All chemicals used were of the highest analytical purity available. Chitin oligosaccharides were prepared by the method of Rupley [8]. Association constants K_a were determined by fluorometric titrations as described by Chipman et al. [9].

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Solutions for the kinetic studies were all in 0.1 M sodium phosphate buffer, pH 8. This pH was chosen since it gives a maximal value of the fluorescence enhancement caused by saccharide binding. Moreover, under these conditions, the rate of hydrolysis of the saccharides is negligible.

The initial enzyme concentrations used varied from 0.001 to 0.07 mM whereas the concentrations of $(GlcNAc)_3$ varied from 0.01 to 0.2 mM, of $(GlcNAc)_4$ from 0.006 to 0.1 mM and $(GlcNAc)_5$ from 0.01 to 0.2 mM. All kinetic measurements were carried out at 25°. An improved model of the temperature jump apparatus was used. This apparatus and the reaction cell were specially designed to follow fluorescence changes due to chemical relaxation [10]. The light source was a 200 W Xenon-Mercury arc. Either a Zeiss M4Q (III) or a Bausch and Lomb monochromator was employed to select the exciting wavelength (285 nm). The fluorescence was observed at 90° to the exciting beam through a cut-off filter. The experiments with iodine oxidized lysozyme were performed under the conditions described by Chipman and Schimmel [3] on a double beam T-jump relaxation spectrophotometer.

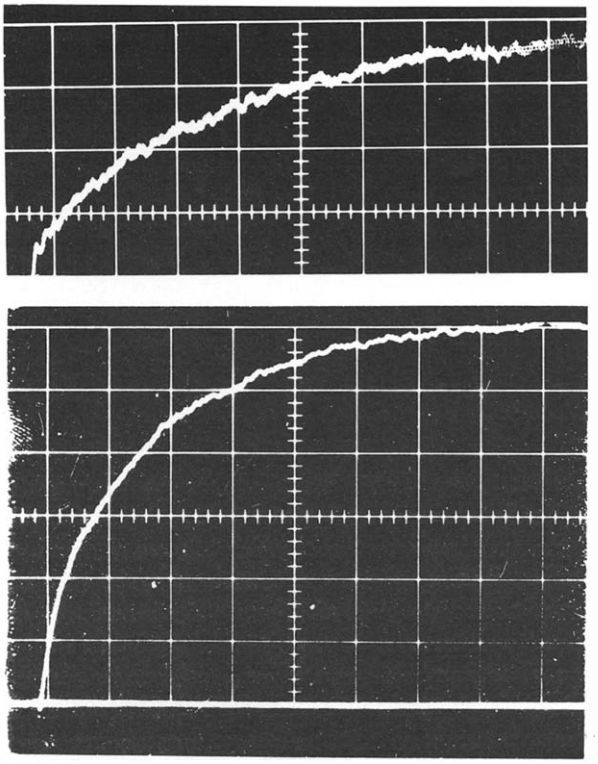

Fig. 1. Oscilloscope traces of the fluorescence changes due to the relaxation of:
a) Lysozyme 1.76×10^{-5} M/l; $(GlcNAc)_5$ 8.02×10^{-5} M/l. Sweep rate: 5 msec/cm (upper trace).
b) Lysozyme 6.8×10^{-6} M/l, $(GlcNAc)_3$ 1.4×10^{-5} M/l. Sweep rate: 10 msec/cm (lower trace). 0.1 M sodium phosphate buffer pH 8. Exitation wave length, 285 nm. Temperature jump of 8° (17° to 25°).

3. Results and discussion

Fig. 1 shows two typical oscilloscope traces obtained from the relaxation of a lysozyme-saccharide complex. With all three chitin oligosaccharides investigated only a single relaxation process was observed in the time range between 100 μsec and 100 msec. No relaxation of the enzyme in the absence of saccharide could be detected. The dependence of relaxation time on the sum of free concentrations of enzyme and saccharides is given in fig. 2. The results obtained with the three compounds may be considered as fitting the same curve. The relaxation time decreases, and eventually tends to become concentration independent, when the free concentrations of enzyme and saccharide further increase.

This behaviour is consistent with the following mechanism [11, 12]:

$$E + S \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} ES_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} ES_2 \qquad (1)$$

which was previously suggested by Holler et al. [5] for the binding of $(GlcNAc)_2$ and $(GlcNAc)_3$ to lysozyme.

In this mechanism, the first step, namely the binding of enzyme and saccharide, is assumed to be a fast one. It is followed by a slow step in which the enzyme-saccharide complex ES_1 is converted by isomerization to ES_2.

The observed relaxation time is that of the second step τ_2 and should obey equation 2

$$\frac{1}{\tau_2} = k_{32} + \frac{k_{23}(\bar{E} + \bar{S})}{\bar{E} + \bar{S} + K_{S_1}} \qquad (2)$$

Table 1
Rate and equilibrium constants for the lysozyme-saccharides binding.

Saccharides	$1/K_{S_1}$	k_{23}	k_{32}	Overall association constant K_a	
				Calculated	Observed
$(GlcNAc)_3$	9×10^3	380	3	1.1×10^6	1.2×10^5
$(GlcNAc)_4$	1.25×10^4	340	2	2.1×10^6	0.9×10^5
$(GlcNAc)_5$	1.25×10^4	340	2	2.1×10^6	1.2×10^5

Constants refer to the mechanism:

$$E + S \underset{}{\overset{K_{S_1}}{\rightleftharpoons}} ES_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} ES_2$$

$K_{S_1} = k_{21}/k_{12}$; Overall apparent association constant: $K_a = 1/K_{S_1}\,(1 + k_{23}/k_{32})$.

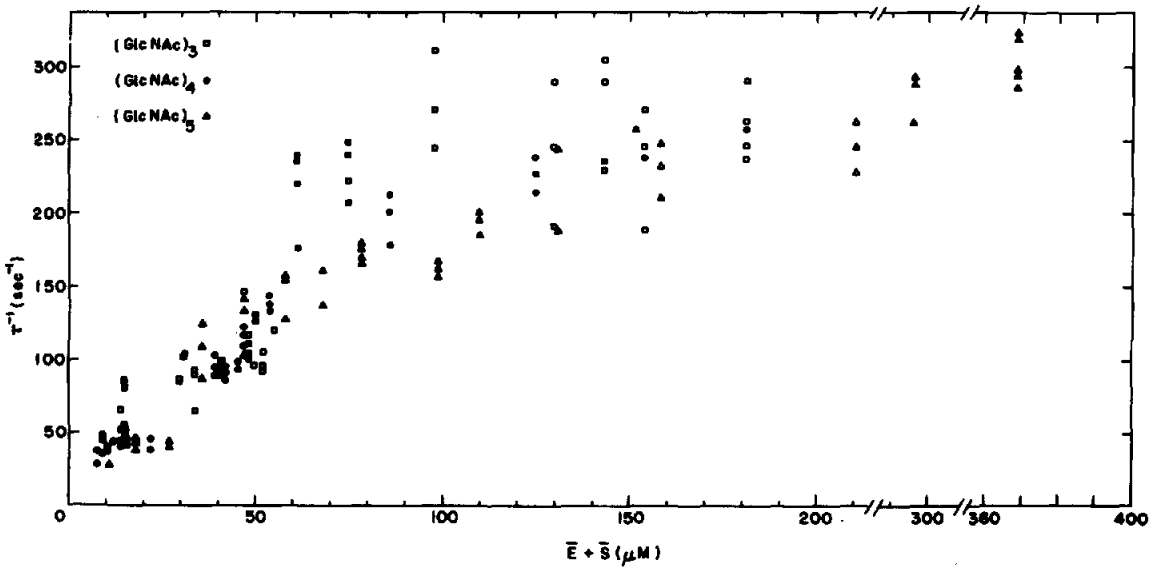

Fig. 2. The reciprocal relaxation time for the lysozyme-saccharide ($(GlcNAc)_{3,4,5}$) complex formation at pH 8 as a function of the sum of the equilibrium free concentrations of enzyme and saccharides. □: $(GlcNAc)_3$; ●: $(GlcNAc)_4$; △: $(GlcNAc)_5$.

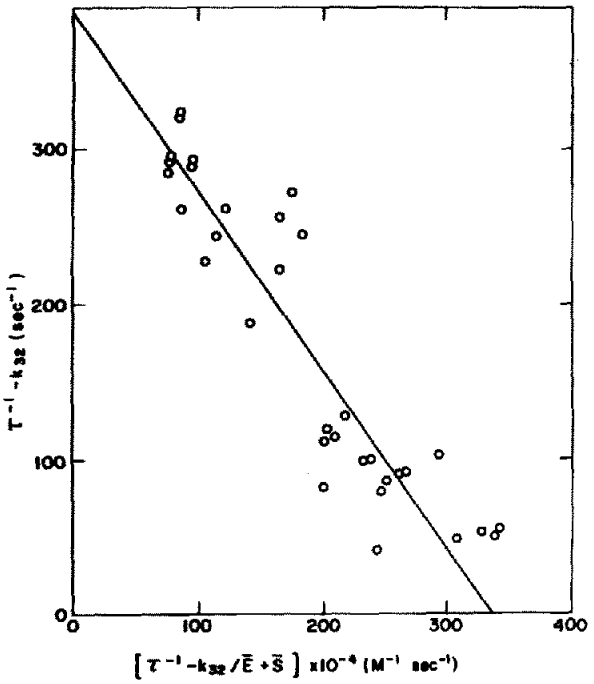

Fig. 3. Data plotted according to equation 3. The points represent the data shown in fig. 2. The line was established by a computer program which calculated k_{32} by iteration to give the best linear fit. The slope and the intercept correspond to K_{S_1} and k_{23}, respectively.

where $K_{S_1} = k_{21}/k_{12}$ and $\bar{E}$ and $\bar{S}$ are the equilibrium concentrations of free enzyme and free saccharide computed from their total concentrations and equilibrium binding constant.

Rearrangement of equation (2) leads to the following expression:

$$\frac{1}{\tau_2} - k_{32} = k_{23} - \frac{\frac{1}{\tau_2} - k_{32}}{\bar{E} + \bar{S}} K_{S_1} \qquad (3)$$

for which K_{32} for each saccharide, was calculated by iteration using an appropriate computer program leading to a best linear fit (fig. 3). From the slope and the intercept of the calculated line, K_{S_1} and k_{23} respectively, were obtained.

The kinetic parameters for the trimer, tetramer and pentamer are nearly identical (table 1). It would be appear, therefore, that both the binding step and the relative stabilities of the two conformations ES_1 and ES_2 are independent of saccharide size for the compounds studies.

The values of the overall binding constants calculated from K_{S_1}, k_{23} and k_{32} (see table 1) differ from the equilibrium binding constants obtained by static measurements. This might be due to the existence of additional slow isomerization step (or steps) which could not be observed experimentally. Such steps were very recently proposed by Owen et al. for the free enzyme [13] and by Holler et al. [14] for the enzyme complex with $(GlcNAc)_6$.

We previously concluded that the indole ring of Trp-108 is not essential for inhibitor binding, even though the fluorescence changes accompanying the process originate from this residue and are caused by an inhibitor induced conformational change [6]. Therefore the isomerization rate constant might represent the rate at which the spatial rearrangement of and around Trp-108 is taking place. Holler et al. [5] have shown that the change in conformation which accompanies saccharide binding involves an ionizable group. In order to obtain information on the nature of this group, we examined the proton uptake reaction of iodine oxidized lysozyme in the presence of $(GlcNAc)_3$ and compared it with that of the native enzyme under the same conditions. No proton uptake could be observed in the iodine oxidized lysozyme–$(GlcNAc)_3$ system. This result can be interpreted in terms of the recent X-ray crystallography studies of iodine oxidized lysozyme [15]. In this study, Blake has shown that iodine oxidation of lysozyme, under conditions which modify only Trp-108, causes a change in conformation which is restricted to the region of this Trp only. The finding that modification of this tryptophan abolishes the proton uptake combined with the fact that glutamic 35 is the only ionizable group in the vicinity of this modified Trp residue, implies that the carboxyl side chain of Glu 35 is responsible for the proton uptake reaction.

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